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TITLE OF THE INVENTION (500 characters max)

Rational Design and Engineering of Proteins and Peptides for Immunomodulation

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Number 1 of 1

Rational Design and Engineering of Proteins and Peptides for Immunomodulation

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ABSTRACT

A novel class of protein mimics is described with the potential to variably modulate the monocyte derived phagocytic cells. These mimics are derived from a naturally occurring serum protein, Vitamin-D binding protein, or Gc-globulin, which after specific deglycosylation is able to activate macrophages, enabling them to remove cancer cells and other foreign particles including bacteria and viruses. The partially deglycosylated variant of Vitamin D binding protein is known as macrophage activating factor (Gc-MAF). We have developed reduced-size polypeptide mimics of Gc-MAF protein. The active site domain of Gc-MAF was altered and optimized using commercially available molecular modeling software. The active amino acid residues were excised and implanted/grafted on a distinct protein scaffold. A peptide mimetic of the sugar residue, N-acetyl-D-galactosamine (GalNAc), which is required for activity, was achieved by screening with a GalNAc-specific lectin. The goal of this work is to develop novel, more stable and effective mimics of Gc-MAF monocyte mediated immunomodulation. The Gc-MAF mimics (from hereon called immunostimulatory peptides, IMPs) are made by chemical synthesis and/or recombinant techniques in living cells and/or cell-free environment. The IMPs were designed with/without amino acid side chain modifications (e.g., post-translational modifications) and are able to activate phagocytosis in a dose dependent manner by the monocyte-derived phagocytic cells. These IMPs can be administered to animal and human subjects to modulate the immune cell activation primarily to combat cancer and infectious diseases.

A. Cancer and Immune System:

The immune system represents the endogenous defense mechanism that constantly scans for 'self' and 'non-self' molecules and organisms in the body. The immune response against 'non-self' entities is initiated upon their encounter with the phagocytic cells, such as macrophages and dendritic cells. The phagocytic cells engulf and digest the foreign substance/cells and display specific antigens on their surface. These antigenic fragments alert the helper T cells to begin a precisely choreographed attack which ultimately results in cellular and humoral immunity against the foreign intruder.

The immune system also recognizes cancer cells as foreign and removes them. However, the fact that this event is rare suggests that either the immune system does not easily distinguish cancer cells from healthy cells and/or cancer suppresses the immune system. Several approaches have been used to stimulate, repair or enhance the immune system, such as specific antibody targeting, lymphokine treatment and infusion of activated dendritic cells[1]. The induction of tumor immunity can be initiated by the effectors of innate immunity and further developed by cells of adaptive immunity, with phagocytic cells such as macrophages and dendritic cells playing a central role in linking these defense mechanisms.

B. Macrophage Activating Factor (MAF):

Biological response modifiers such as immune stimulators, lymphokines, antibodies and specific carbohydrate epitopes activate the immune system to recognize cancer cells. Macrophage activating factors activate macrophages and allow them to act

as cytotoxic cells that non-specifically kill tumor cells. MAF is more or less an operational definition for a particular biological activity rather than the name of a distinct factor. One of the macrophage activating factors is derived from vitamin-D binding protein (VDBP), also known as Gc-globulin, and is called Gc-MAF. Gc-MAF is an abundant serum glycoprotein composed of three domains. The C terminal domain III contains 120 amino acids and is crucial for macrophage activation. Domain III of precursor Gc-MAF (VDBP) is post-translationally O-glycosylated at threonine 420 with an oligosaccharide moiety composed primarily of N-acetyl-D-galactosamine (GalNAc), galactose and sialic acid residues[2]. Activation of Gc-MAF is accomplished by selective removal of sugars by galactosidase and sialidase present on B- and T-cells, respectively[3] (Fig. 1). A single GalNAc residue is retained, and mediates the interaction of activated Gc-MAF with a C-type lectin receptor on the macrophage surface[4; 5]. This interaction results in macrophage activation for phagocytosis and subsequent antigen presentation.

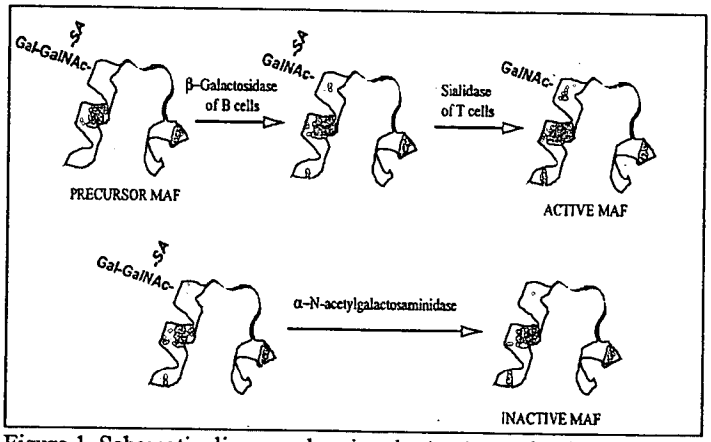

Figure 1. Schematic diagram showing the *in vivo* activation of MAF by selective deglycosylation by β-galactosidase and sialidase and its inactivation by N-acetyl galactosaminidase.

However, cancerous cells secrete an enzyme, α-N-acetyl-D-galactosaminidase (GalNAcase) into the blood stream, which results in complete deglycosylation of serum Gc-MAF leading to immunosuppression[6; 7]. It has been shown that the administration of enzymatically activated Gc-MAF to mice will overcome the inactivation and result in macrophage activation. These observations show that Gc-MAF can be useful as an adjuvant to enhance and accelerate the development of the immune response and to generate a large amount of antigen-specific antibodies. More importantly, systematic and repeated application of activated Gc-MAF has been shown to be effective as a general therapy for cancer and viral diseases.

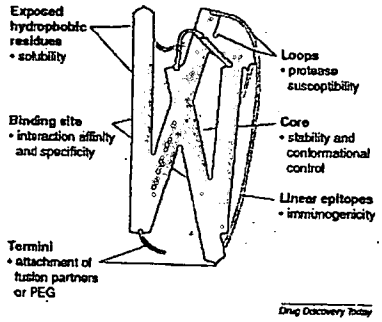

Fig. 2: Strategies for rational design and protein engineering

Until recently, mammalian serum has been the only source of Gc-MAF restricting its applications to patients. Concerns regarding potential contamination with infectious agents preclude obtaining Gc-MAF from this source. In addition, the serum-derived Gc-MAF has a molecular mass of ca. 52 kDa and carries only one active site (Thr-GalNAc); thus a large amount of this protein is required for therapeutic effects. The requirement for glycosylation restricts production of the full-length or derivative proteins to other eukaryotic systems.

C. Rational Protein Drug Design:

In recent years, rational protein design has proven to be a valuable tool for optimizing therapeutic proteins[8; 9]. This approach enables researchers to design and develop macromolecular drugs with specific physical and biological attributes. ***Several engineered proteins obtained by rational design are currently on the market or have completed clinical trials, generating a revenue of approximately US$ 30 billions in 2001.*** Examples of engineered protein therapeutics are HumaLog® (Eli Lilly) and NovoLog® (Novo Nordisk), fast-acting versions of insulin; Ontak® (Seragen), a natural toxin reengineered to target cancer cells; Fuzeon® (Trimeris), an inhibitor of HIV fusion derived from the viral protein gp41. In these drugs, properties such as activity, stability, solubility, specificity, immunogenicity and pharmacokinetics have been successfully optimized (Fig. 2). Starting from the detailed knowledge of the protein structure, rational design[10; 11] involves computational simulations and evaluations of a library of *in silico* mutants that are ultimately prepared and screened for activity *in vitro* and *in vivo*. The computational simulation steps allow the narrowing of the choice of protein variants to a manageable number. In contrast, experimental methods to create library of mutants rely on the randomization of residues at selected positions and results in extremely large libraries, which can be challenging to screen experimentally. For example, if one randomly mutates 10 residues in a protein to each of the 20 possible amino acids, the size of the library will be 20^{10}! Rational design allows to pre-screen a large number of mutants *in silico* and to restrict the size of the experimental library to a few mutants. In a second step, the experimental data obtained on each mutant can be utilized for the design of second-generation optimized proteins. This last step is conceptually similar to traditional Quantitative Structure-Activity Relationships (QSAR) methods, but utilizes protein-specific computational methods[12; 13].

The developing field of peptide mimetics has also extended to non-amino acid structures. It has been shown that peptide sugar mimetics can induce functional carbohydrate cross-reactive immune responses in pathogen and tumor models[14]. Random peptide libraries displayed on phage provide a starting material for a number of applications in protein chemistry, including identification of peptide mimics of non-peptide ligands[15]. With this approach, a mimetic of GalNAc has been identified.

D. Structural Optimization and Synthesis of IMPs:

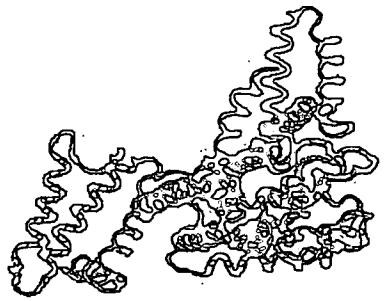

Fig 3. Crystal structure of MAF. Domain III is highlighted in blue

Domain III of Gc-MAF is the site of the specific glycosylation event that leads to its bioactivation. In broader terms, a domain of a protein is an independently folded unit that can be separated from the intact protein and retain a specific structure. Domains often serve as the smallest functional elements of a complex protein, in which two or more domains can be combined to obtain complex functions. For example, Gc-MAF contains a vitamin D-binding domain, an actin-binding domain, and the glycosylation site, Domain III, which is crucial for macrophage

activation[16]. The use of Domain III in lieu of full length-Gc-MAF in therapy would present several advantages: first, the size of the isolated Domain III, ca. 120 amino acids, would make it a more tractable drug; second, by dissecting the desired function one would avoid possible cross-reactivity and side effects. However, preliminary studies show that the activity of the isolated Domain III is significantly reduced in comparison to the full length-Gc-MAF activity[7]. A possible explanation is given by visual inspection of the crystal structure of Gc-MAF[17; 18]: Domain III is a distorted three-helix bundle, and makes significant hydrophobic and ionic contacts with the remaining Gc-MAF (Fig. 3). It is conceivable that the isolated Domain III would not be sufficiently stable under physiological conditions.

We are using a rational design approach to prepare optimized, miniaturized proteins as mimetics of Domain III. A similar approach was pioneered by J. Wells' group at Genentech in the design of Protein A analogs[19; 20], and has recently been used to design analogs of tDNA-binding domains, apoptotic proteins[23; 24], and of the CD4 receptor[25; 26]. Because of the distorted three-helix bundle topology of Domain III, we decided to use a stable peptide scaffold of similar but more regular topology as a starting point. The putative active site of Domain III, defined as the portion of the protein surrounding the glycosylation site, was grafted onto a stable three-helix bundle scaffold obtained form the Protein Data Bank[27] and the resulting model protein optimized as described in Preliminary Studies.

The choice of the scaffolds was guided by three considerations: first, the size is considerably smaller than Domain III, and well within the limits for solid-phase synthesis of peptides; second, the scaffold is well characterized in terms of its stability and biophysical properties; third, the scaffold is amenable to structural studies. This approach has the advantage of starting from a structured template with minimal sequence homology to the native protein, thus avoiding possible interferences with undesired functions of the native protein, or sensitivity to proteases, which would be an important consideration in the *in vivo* use of biomimetics. Incorporation of the peptide mimetic of GalNAc into the peptide sequence provides the required recognition site at no additional cost and creates a structure that cannot be inactivated by glycosylases.

E. Protocol for IMPs Optimization:

In order to design an optimized version of Domain III, we used computer graphics to identify the critical residues for activity. Specifically, the glycosylated threonine 420 is located in a solvent-exposed loop, and protrudes from the start of one of the helices; the sequence surrounding Thr 420 is likely recognized by glycosylation enzymes and by the specific receptor located on the

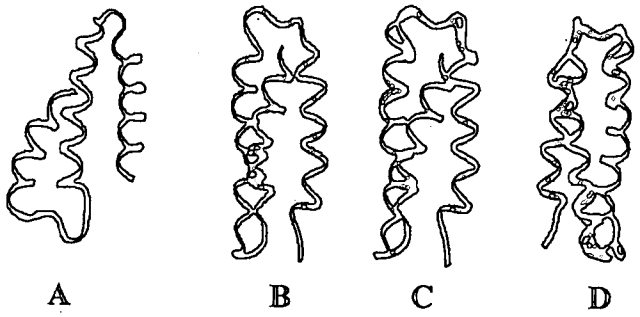

Fig. 7: Comparison of Domain III (A), the scaffold 1LQ7 (B), superimposition of the loop of Domain III onto one of the loops of 1LQ7 (C), and superimposition onto both loops of 1LQ7 (D).

surface of the macrophages. Thus, we narrowed the putative active sequence to approximately 20 residues, which comprise the loop and the first turn of the α-helix on each side. Using Insight II, a molecular modeling software package, we investigated the stability of the isolated 20 amino acid sequence by running energy minimization experiments. The results show that the minimized loop presents severe deviations from the three-dimensional structure assumed in the native protein. Clearly, the underlying three-helix structure of Domain III is critical to restrain the conformation of the loop to the biologically active form. Therefore, we decided to transfer the active loop of Domain III in the native conformation onto a more stable three-helix bundle obtained from the Protein Data Bank. The scaffold chosen, 1LQ7, was originally designed in Prof. P.L. Dutton's lab at the University of Pennsylvania, and has the additional advantage of being amenable to solid-state synthesis[27].

We started by aligning the sequence of the 20 amino acid loop with that of the scaffold, using the position of the helical residues on each side of the loop as guide. We overlaid the aligned coordinates of the loop onto those of the template (Fig. 7), obtaining a remarkable superimposition of the two structures. The scaffold loop was then replaced with that of the Domain III; a few alternative fragment lengths were tested for the substitution. In order to increase the protein activity, both scaffold loops were replaced with Domain III amino acids to yield a bifunctional molecule. The 70 amino acid constructs retain the overall three-helix bundle topology of Domain III, in a more regular and stable version. Each version was optimized by energy minimization routines and evaluated to identify significant deviations from the native loop conformation. The model that showed the smallest deviations from the native conformation was selected and will be the starting point for protein optimization. The amino acid sequence of the 1LQ7 scaffold with reengineered Gc-MAF loops is GSRVKALEEK VKALEEKVKA L*PNATPTELAK* KKWEELKKK IEELG *NATPTE* VKKVEEEVKKL EEEIKKL (the active amino acids from the natural Gc-MAF molecule are shown in bold).

F. Construction of a GalNAc Mimetic

Combinatorial peptide libraries displayed on phage M13 provided approximately 3×10^9 variants of a 12-amino acid sequence. Each of these sequences were present in about 55 copies. The total library was screened with a GalNAc-specific lectin to search for a peptide mimetic of the sugar. Phage particles that bound to the lectin were eluted with free GalNAc and the process was repeated several times. After DNA of phage that were selected by this procedure was sequenced, a consensus amino acid sequence emerged. Insertion of variations of this sequence should allow construction of a series of IMPs with which binding affinities and consequently activity can be manipulated to achieve optimal clinical results. [Commercialization of sequences discovered using Ph.D.™, a trademark of New England Biolabs, Inc., may require a license from Dyax Corp.]

G. Additional Activities

- We are identifying the minimal active site of Gc-MAF by systematically altering the loop residues to achieve the desired biological activities.
- Glycosylation of the IMPs will be achieved by either incorporating glycosylated amino acids or by in-vitro glycosylation reactions.

- Both hydrolyzable and non-hydrolyzable linkage between the sugar residue and the amino acids are being designed.
- The IMPs will be PEGylated for longer circulatory half-life in-vivo.

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